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Monocyte activation by apoptotic cells removal in systemic lupus erythematosus patients

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ABSTRACT

Decreased apoptotic cells (ACs) removal has been described as relevant in systemic lupus erythematosus (SLE) pathogenesis. Binding/phagocytosis of ACs was decreased in SLE patients. Blocking experiments suggested a role for CD36 in ACs clearance in healthy controls, not observed in SLE patients. Binding/phagocytosis of ACs induced the production of IL-6, CXCL8 and CCL22 in patients and controls and IL-1β, TNF-α and CCL3 only in healthy controls. ACs clearance induced an increase in CD80 and a decrease in CD86 expression in healthy controls and atherosclerotic patients. However, SLE patients did not up-regulate CD80 expression. The number and expression of CD36 and CD163 in monocytes was not different between the groups. ACs removal induced a down-regulation of CD36 expression in adherent HLA-DR$^+$ cells in SLE patients but not healthy controls. The decreased binding/phagocytosis of ACs observed in SLE patients, induces a distinct immune response compared with healthy controls.

1. Introduction

Patients with SLE have increased circulating apoptotic cells (ACs), possibly due to the accelerated apoptosis of lymphocytes [1] and decreased AC clearance [1,2]. AC binding/phagocytosis can be mediated by different receptors present on the surface of monocytes/macrophages, neutrophils and dendritic cells, such as CD36, vitronectin receptor ($\alpha_v\beta_3$), thrombospondin [3,4], CD14, FcR and complement receptors [5].

Whether or not phagocytosis of ACs induces an inflammatory or anti-inflammatory response is still controversial and it may depend on the immunologic milieu in which this process occurs. The production of IL-10 and TGF-β anti-inflammatory cytokines and inhibition of IL-12 and TNF-α pro-inflammatory cytokine production has been previously reported [6], whereas other studies report pro-inflammatory outcomes and enhanced immune responses after exposure to ACs [7–9].

These differences might be explained by the way apoptosis was induced. Previous reports have shown the production of inflammatory molecules after phagocytosis of ACs induced by γ-radiation [10] or by deprivation of growth factors [9]. However, other reports support that the method used to induce apoptosis is irrelevant [6,11]. These differences could also be explained by the different experimental approaches used, since the studies differed in the type of phagocytic cells. Some investigators evaluated AC removal by macrophages in mice models exposed to high ionizing radiation [10], whereas others performed ex-vivo assays with phagocytic cell lines and human monocytes [9].

In addition, the induction of apoptosis with UVB radiation and its removal in SLE patients has been associated with the production of inflammatory cytokines [12–14]. Furthermore, AC clearance has consequences on cytokine production and on the modulation of chemokine receptors and costimulatory molecule expression [15].

Among the receptors involved in AC clearance are CD36 and CD163 scavenger receptors [16]. AC clearance mediated by CD36 has been reported to induce an anti-inflammatory response. This was shown by the incubation of monocytes with an agonist anti-CD36 antibody that mimicked the anti-inflammatory effects of UV-induced ACs, inducing the production of TGF-β and IL-10 and decreasing TNF-α, IL-1β and IL-12 production [6,17]. In addition, the high expression of CD163 has been proposed as a marker of the alternative macrophage activation, which is associated with dampening the inflammatory response [18].

CD36 and CD163 are also involved in oxidized low density lipoprotein clearance [16,19,20] and in the onset of the atherosclerosis process [19,21,22]. Atherosclerosis is nowadays recognized as an inflammatory disease with high prevalence in autoimmune diseases, particularly prevalent in SLE [23]. In a murine model of SLE and atherosclerosis (*gld.apoE*$^{-/-}$) it was shown that a decreased

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AC clearance plays an important role in the development of atherosclerosis [24].

In the present study, we aimed to evaluate the consequences of AC clearance by monocytes from SLE and atherosclerotic patients and healthy controls. Results show that the binding/phagocytosis of UV-induced ACs by $CD14^+$ cells induced the production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and the chemokines CCL3, CXCL8 and CCL22 in healthy controls and IL-6, CXCL8 and CCL22 in SLE and atherosclerotic patients. SLE patients, compared to healthy controls, showed differential modulation of CD80 and CD86 costimulatory molecules, and decreased binding/phagocytosis of ACs that is not CD36- and CD163-dependent. These findings may be related to the increased immune activation and decreased tolerance observed in SLE patients.

2. Materials and methods

2.1. Reagents

RPMI-1640, PBS and Fetal Calf Serum (FCS) were purchased from GIBCO-BRL (Grand Island, NY.); penicillin, streptomycin, Ficoll-Hypaque, Pooled Human AB Serum (PHS) and Limulus Amebocyte Lysate (LAL) from Cambrex-BioWhittaker (Walkersville, MD); trypan blue, sodium azide and bovine serum albumin from SIGMA (Saint Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from MERCK (Darmstadt, Germany). Ethidium bromide (EtBr) and 3,3′-dihexyloxacarbocyanine iodide (DIOC6) were purchased from Invitrogen (Carlsbad, CA). 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR) and CALTAG Cal-Lyse Lysing solution from Caltag Laboratories Inc. (Burlingame, CA). Monoclonal anti-CD14-FITC (clone M5E2), anti-CD36-PE (clone CB38), anti-CD163-PE (clone GHI/61), anti-HLA-DR-Cy (clone TÜ36), anti-CD14-PE (clone M5E2), anti-CD80-PE (clone L307.4), anti-CD86-PE (clone IT2.2), anti-CD40-PE (clone 5C3), isotype controls $IgG2a_k$-FITC (clone G155-178), $IgG2a_k$-PE (clone G155-178), $IgG1_k$-Pe (MOPC-21), IgM_k-PE (clone G155-178), $IgG2b_k$-PE-Cy5 (clone 27-35), the blocking anti-CD36 (clone CB38), anti-CD163 (clone 6HI/61), polymethylmethacrylate beads, 7-AAD BD via-probe cell viability solution, 6-well flat-bottom plates and the Human Inflammation Kit-CBA were all purchased from BD Bioscience (San Jose, CA). Annexin V-PE Apoptosis Detection Kit 1 was purchased from BD Pharmingen (San Diego, CA). Detection and capture anti-CCL2, anti-CCL22, anti-CXCL8 and anti-CCL3 antibodies for ELISA were purchased from R&D Systems (Minneapolis, MN). Acetic acid was obtained from Mallinckrodt (Hazelwood, MO). Twenty-four well flat-bottom culture plates were obtained from Greiner Bio-One (Stuttgart, Germany). All media were negative for LPS as evaluated by LAL assay.

2.2. Patients and controls

Thirty-four patients diagnosed with SLE (36 females/1 male) according to the American College of Rheumatology Criteria [25], 20 atherosclerotic patients (20 females/0 male) with confirmed previous atherosclerotic events (myocardial infarction, stroke or acute limb ischemic event) without autoimmune disease, and 29 healthy controls (28 females/1 male), were included in this study. Mean ages were 34 ± 13, 61 ± 9 and 28 ± 8, respectively. Patients with SLE were classified by the systemic lupus erythematosus disease activity index (SLEDAI) [26] in active ($\geqslant 8$) ($n = 16$) and non-active (<8) ($n = 18$) with a mean ± SD SLEDAI of 12 ± 10. SLE patients were under different immunosuppression schedules with prednisone, chloroquine, cyclophosphamide, mycophenolate mofetil and azathioprine. Atherosclerotic patients were also under different treatments with captopril, metoprolol, warfarin, ASA and statins. All patients and controls signed an informed consent previously approved by the Ethics Committee of the Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia.

2.3. CFSE staining of Jurkat cells and apoptosis induction

Jurkat cells (human T cell line, kindly provided by Dr. Augusto Ochoa, Louisiana State University, New Orleans, LA) were stained with prewarmed 1 μM CFSE in PBS for 15 min at 37 °C in the dark, and incubated for 30 min in complete medium (RPMI-1640 plus 10% heat-inactivated FCS, penicillin-100 U/ml, streptomycin-100 μg/ml) at 37 °C in a water bath, washed and resuspended in PBS. More than 90% of the cells were viable, as confirmed by trypan blue exclusion, and 90–97% of the cells were stained with CFSE as evaluated by flow cytometry. Five million CFSE-labeled Jurkat cells were resuspended in 4 ml of PBS in 6-well flat-bottom culture plates and exposed to UV light (110 mJ-65 s) in a UV linker oven (GS Gene linker™ UV Chamber, Bio Rad, Hercules, CA). Then, cells were cultured under starvation conditions (PBS) for different times (24, 48 and 72 h) followed by centrifugation on Ficoll-Hypaque at 400*g* for 30 min at room temperature (RT). Viable cells were collected from the interface and ACs from the bottom. ACs were washed twice with PBS and frozen in heat-inactivated FCS plus 10% DMSO. Apoptosis was established by flow cytometric evaluation of size (FSC) and granularity (SSC). This mainly showed the presence of small cells with increased granularity (Fig. 1A, R6), which corresponds to the morphology of apoptotic cells [27]. An aliquot of unstained ACs was also evaluated for mitochondrial and extracellular membrane damage by the staining with DIOC6 (7 μM) and EtBr (1.3 mg/ml) by flow cytometry. Around 80% of the cells were DIOC6 low and EtBr negative, suggesting that mitochondrial permeability was altered in these cells, but their plasmatic membrane was intact (Fig. 1B). Annexin V-PE and 7-Actinomycin D (7-AAD) staining were also carried out. Briefly, 1×10^6 frozen CFSE-labeled Jurkat cells induced to apoptosis were incubated with 5 μl of Annexin V-PE or 7-AAD for 20 min at RT, and were then washed either with Annexin buffer or with PBS and analyzed by flow cytometry. Around 80% of the cells were also positive for Annexin V-PE (Fig. 1C) and negative for 7-AAD (Fig. 1D), confirming that cells were apoptotic.

2.4. Binding/phagocytosis assays

PBMCs from patients and controls were isolated by centrifugation on Ficoll-Hypaque and frozen in liquid nitrogen until used. Five hundred thousand (5×10^5) PBMCs were thawed and $CD14^+$ cell number was established by flow cytometry. Thereafter, PBMCs were incubated with different ratios of CFSE-labeled ACs (4, 6 or 10 ACs) per $CD14^+$ cell in a final volume of 1 ml of complete medium for 1 h at 37 °C, 5% CO_2, 90% humidity in flow cytometry polystyrene tubes (BD-Falcon, San Jose, CA). Thereafter, cells were washed, incubated for 10 min at RT, stained with 5 μl of anti-CD14-PE for 30 min at 4 °C and analyzed by flow cytometry. Binding/phagocytosis of CFSE-ACs was analyzed on the $CD14^+$ cell gate.

2.5. Evaluation of CD36 and CD163 expression and effect of CD36 and CD163 blockade on AC phagocytosis

For ex-vivo evaluation of CD36 and CD163 expression on monocytes, 100 μl of EDTA-anticoagulated blood from patients and controls were mixed with anti-CD14-FITC (15 μl) and anti-HLA-DR-Cy (10 μl), plus anti-CD163-PE (10 μl), anti-CD36-PE (6 μl) or isotype control antibodies (IgG2aκ-FITC, IgG1κ-PE, IgMκ-PE and IgG2bκ-PE-Cy5) and incubated for 20 min in the dark. Then 100 μl of Caltag buffer were added to each tube and incubated for 10 min under the